Determination of intact zeatin nucleotide by direct chemical ionisation mass spectrometry

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1. INTRODUCTION

9-β-D-Ribofuranosylzeatin 5′-monophosphate (ZMP) has so far been isolated in crystalline form and identified unambiguously from *Zea mays* kernels only [1]. Identification was based principally on enzymic and chemical degradation. However, in a number of plant tissues, nucleotides of zeatin, dihydrozeatin and isopentenyladenine have been identified, by chromatographic methods, as metabolites of exogenous cytokinins, and cytokinin nucleotide formation may be associated with cytokinin uptake [2]. More recently, cytokinin nucleotides have been identified as the primary products of cytokinin biosynthesis [3–5]. Indeed, cytokinin nucleotides appear to play a central, and possibly a key, role in cytokinin metabolism [6].

However, in the past, most cytokinin isolation and characterisation procedures have tended to concentrate on the cytokinin bases and their glycosides which are normally purified by retention on cation exchange (preferably cellulose phosphate) columns. Surprisingly little effort has been made to identify and quantify cytokinin nucleotides present in the anionic fraction not retained by such columns. Nucleotide analyses utilising mass-spectrometry (MS) and gas chromatography (GC)–MS usually require a dephosphorylation step with alkaline phosphatase. However, this approach does not distinguish between the following compounds unless these compounds are separated before dephosphorylation: cytokinin riboside 3′- and 5′-mono-, and the corresponding di- and tri-phosphates and isomeric compounds in which the phosphate is attached to the oxygen on isopentenyl side chain. Chromatographic methods for this separation have not been established since the relevant markers have not been synthesized. We now report a facile method to obviate some of these problems. It utilizes direct chemical ionization (CI) mass-spectrometry for detection and quantification of intact ZMP in the anionic fraction (fraction not retained by a cellulose phosphate column) of *Datura innoxia* crown-gall tissue extracts. Cytokinin bases and glycosides can be determined simultaneously in the cationic fraction retained by the column as in [7,8].

In [9], a mass-spectrometric method for quantifying ZMP was devised which involves dephosphorylation prior to GC–MS.

Mass spectrometric analysis of cytokinins in plant tissues: IV

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2. MATERIALS AND METHODS

2.1. *Synthesis of nucleotide standards*

(*E*)-4-Amino-2-methylbut-2-enol and its pentadeuterium (D_5)-labelled analogue were synthesised as in [10,11]. 6-Chloropurine riboside-5′-monophosphate (Sigma, 50 mg), methanol (3 ml), triethylamine (50 μl) and the appropriate amine (20 mg) were heated together for 4 h at 90°C. Purification of the products to homogeneity was

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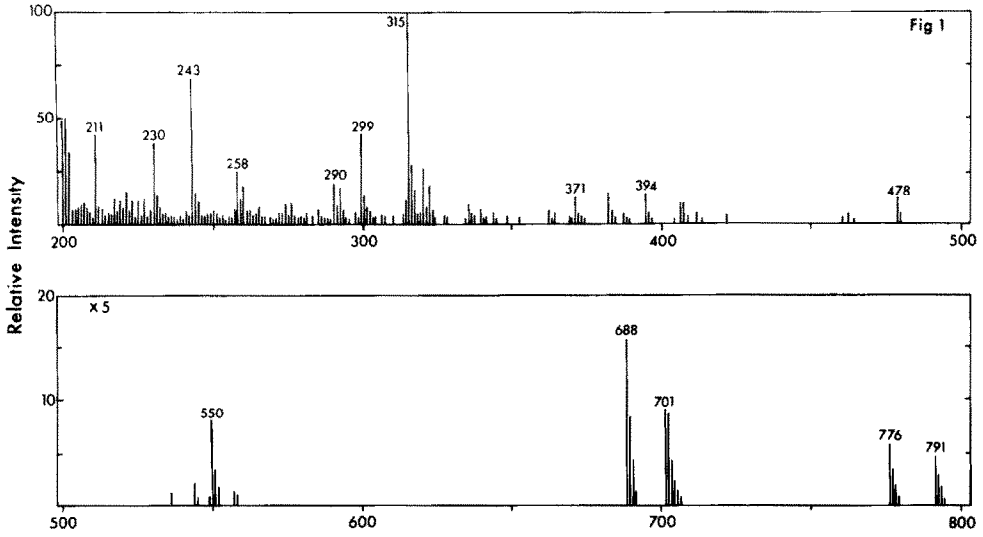

Fig.1. EI mass spectrum of the TMS-derivative of synthetic ZMP.

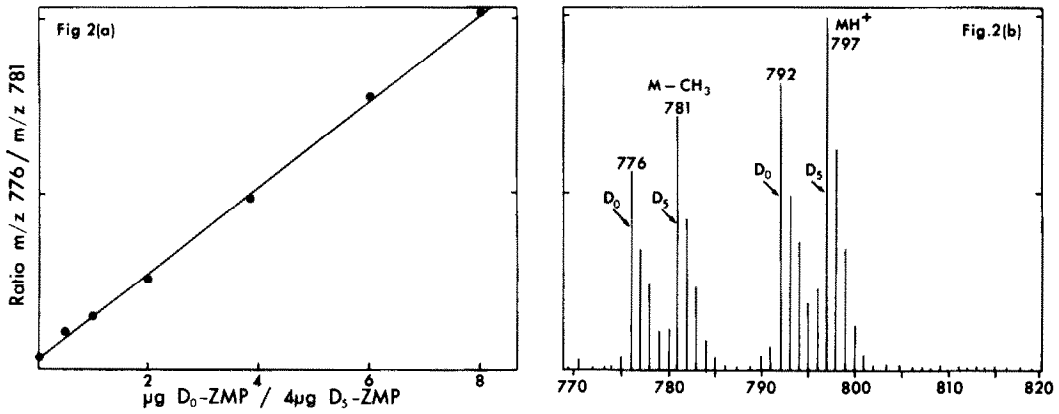

Fig.2.(a) Calibration line for the M–CH_3· ion of D_0 + D_5 TMS–ZMP prepared from UV calibrated standard solutions; (b) Molecular ion region in the CI mass spectrum of TMS–ZMP from the tissue extract.

achieved by separation on Dowex-1 (formate) using 2 N formic acid as eluent, followed by preparative high pressure liquid chromatography on µBondapak C_{18} eluting isocratically with 10% methanol containing 1% acetic acid. The electron impact (EI) mass spectrum of the penta-trimethylsilyl (TMS)-derivative of the unlabelled product is shown in fig.1 and all major ions can be rationalised as arising from the target molecule [12]. The D_5-nucleotide yielded an identical mass spectrum except for the anticipated mass shifts due to D_5-labelling. Furthermore, the unlabelled and D_5-nucleotide co-chromatographed during high pressure liquid chromatography and thin layer chromatography.

2.2. *Chemical ionisation mass-spectrometry and calibration of the labelled standard*

Samples of the nucleotide (1–5 µg) were derivatised using pyridine (5 µl) and BSTFA–TMCS (99:1, 5 µl) and heating to 80°C for 10 min. An aliquot (0.2–0.5 µl) was applied to the direct expo-

sure probe (heatable filament) or to a Vespel probe extension fitted to a Finnigan 4500 GCMS. On contact with the reagent gas plasma (methane, 1 Torr, 190°C), the sample desorbed completely over 20 sec during which the mass-spectrometer scanned from 770–820 a.m.u./0.1 s. A more intense, faster (2-s) desorption could be achieved by supplementary heating of the probe filament. Spectra were integrated over the desorption profile and the area ratios *m/z* 792:797 (MH^+) or *m/z* 776:781 ($MH^+ - 16$) were used for quantification. The latter gave more reproducible ratios (± 0.5% for ratios of 1–10) presumably because of slight variations in the efficiency of protonation.

Standard solutions of the D_5-labelled and unlabelled nucleotide were calibrated by their absorbance at λ_{max} 268 nm. The D_5-solution, for use as the internal standard, was mixed with varying aliquots of the unlabelled solution and the resulting samples used to construct the calibration graph shown in fig.2(a).

2.3. *Extraction of plant tissue and isolation of cytokinin nucleotides*

D. innoxia crown-gall tissue was grown in dark at 25°C on hormone-free B_5 medium [13], solidified with 0.8% agar. Four weeks following subculture the callus pieces were removed and extracted sequentially with $CHCl_3$–MeOH–HCOOH–H_2O (5:12:1:2) [14] and MeOH–HCOOH–H_2O (6:1:4) after enzyme inactivation at −20°C as in [15]. An aqueous solution (pH 3.1) of the evaporated extract was passed through a cation-exchange column (cellulose phosphate, Whatman P1 floc type, 15 ml bed vol. g^{-1} tissue, equilibrated to pH 3.1 in the NH_4^+ form). The H_2O (pH 3.1) wash from the columns was collected, concentrated and loaded onto a DEAE cellulose column (Whatman DE1 coarse fibrous, 10 ml bed vol. g^{-1} tissue, equilibrated to pH 8.0 in the HCO_3^- form). The column was washed with H_2O and the nucleotides eluted with NH_4HCO_3 solution (10%, w/v). The eluate was evaporated to dryness, and freed of bicarbonate by repeated addition and evaporation of MeOH. The sample was then divided into two parts. One portion was treated with *E. coli* alkaline phosphatase [15] and the resulting ribonucleosides were extracted with butan-1-ol and further fractionated on a column of Sephadex LH-20 [3]. Thirty-five fractions of 30 ml were collected, and an aliquot from each fraction was withdrawn for soybean callus bioassay [16]. The main peak of biological activity eluted at the elution volume of zeatin riboside (ZR) and dihydrozeatin riboside (DZR; not shown). These fractions were combined, known amounts of D_5-ZR and D_5-DZR added, and purified by high pressure liquid chromatography (Waters C_8 radial compression cartridge, 100 × 8 mm; linear gradient of methanol, 10–60% in 30 min at 3 ml.min^{-1}, containing 1% acetic acid; rate (min) for ZR = 13.8, DZR = 16.2) [7]. ZR and DZR, which are resolved completely by high pressure liquid chromatography, were collected individually and further analysed by EI and CI GC–MS as TMS-derivatives [11].

To the other half of the sample, known amounts of D_5-ZMP and ^{3}H[AMP] (20000 dpm) were added. The sample was applied to a second DEAE column (pH 8.0 in the formate form) which was washed with H_2O (3 column vol.) and then eluted sequentially with 0.2, 0.5 and 2 N formic acid (2.5 column vol. of each). Fractions containing [^{3}H]AMP were combined and subjected to high pressure liquid chromatography (Waters μBondapak C_{18} column, 300 × 7.8 mm; isocratic elution at 3 ml.min^{-1} with 15% methanol containing 1% acetic acid; rate (min) for ZMP = 9.5). ZMP peak was collected for further analysis by CI–MS as described above.

3. RESULTS AND DISCUSSION

Zeatin nucleotide and its D_5-labelled analog were synthesised from well characterised starting materials and their TMS-derivatives gave EI mass spectra which showed fragmentation patterns consistent with the correct structures. The deuterium-labelled ZMP (8 μg) was subsequently added to a partially purified extract from *D. innoxia* crown-gall tissue and the nucleotides were recovered by ion exchange chromatography on DEAE cellulose. The fraction containing mononucleotide was recognised using tritium-labelled AMP as a marker and further purified by high pressure liquid chromatography. The fraction expected, by its retention time, to contain ZMP was then converted to a TMS-derivative and analysed by direct-exposure chemical-ionisation mass-spectrometry. The resulting mass spectrum is shown in fig.2(b), with

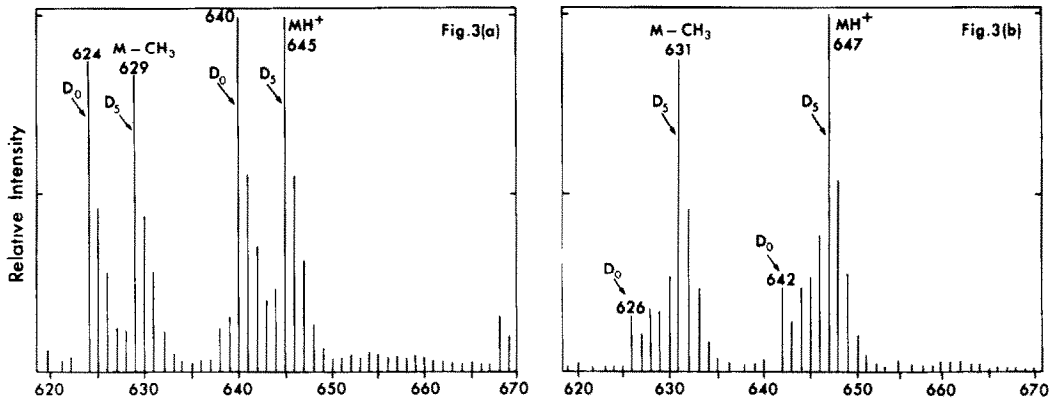

Fig.3. Molecular ion regions of the CI mass spectra of (a) TMS–ZR and (b) TMS–DZR, showing unlabelled endogenous material with the D_5 internal standards.

the D_0/D_5 ratio indicating that the extract from 100 g tissue contained about 6.4 μg of unlabelled, i.e., naturally occurring, ZMP. While TMS–ZMP gives EI and CI mass spectra using a conventional solid probe insertion technique, the 'direct exposure' method offers several advantages. In the former, vaporisation of the sample outside the ion source requires significant heating with consequent thermal degradation and lower sensitivity. However, direct exposure of the sample to the CI reagent gas results in rapid desorption of the desired derivative with minimal decomposition. Supplementary heating causes a very rapid desorption, akin in duration and intensity to a GC peak, with nanogram quantities of substance detectable. Chemical ionisation results in the ion current being concentrated in a few ionic species of relatively high mass (MH^+ 792) and for this plant tissue at least, the presence of impurities did not interfere with detection of the desired compound.

For confirmation of the above observations, an aliquot of the bulk nucleotide fraction eluted from DEAE-cellulose was subjected to alkaline phosphatase hydrolysis and the biologically active products combined with known amounts of D_5-ZR and D_5-DZR [11]. These compounds were subsequently purified by high pressure liquid chromatography and examined by EI GC–MS as described above. The mass spectra showed the expected molecular ion and fragment ion peaks of the deuterium-labelled markers, and superimposed were the corresponding ions of unlabelled, i.e., naturally occurring, ZR and DZR. Under chemical ionisation conditions, the molecular ion regions were as shown in fig.3(a) for ZR and fig.3(b) for DZR. Thus zeatin nucleotide and dihydrozeatin nucleotide were both detected as their ribosides. In addition zeatin was identified subsequent to chemical degradation [17] of the zeatin mononucleotide fraction confirming that the nucleotide was in fact a 5′-phosphate.

In summary, 9-β-D-ribofuranosylzeatin-5′-monophosphate has been detected and quantified for the first time by mass-spectrometry as an intact molecule. This observation was confirmed by simultaneously quantifying the riboside obtained by phosphatase hydrolysis. This approach also led to the identification of 9-β-D-ribofuranosyldihydrozeatin derived from its nucleotide precursor(s). Chemical ionisation mass-spectrometry using the direct exposure probe technique, combined with the use of a deuterium-labelled internal standard, is a sensitive and precise method for observing and quantifying intact cytokinin nucleotide derived from plant tissue.

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